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Evaluation of the Bacteriophage MAC-1 Potential to Control *Pseudomonas aeruginosa* Planktonic Cells and Biofilms

(Penilaian Potensi Bakteriofag MAC-1 untuk Mengawal Sel Plankton *Pseudomonas aeruginosa* dan Biofilem)

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ABSTRACT

Pseudomonas aeruginosa is a pathogenic bacterium that can be considered a high risk to human health due to its remarkable capacity to resist antibiotics, either intrinsically or following the acquisition of resistance genes. *P. aeruginosa* has been considered a major threat to human health as all the known remedies seem ineffective. Bacteriophages as a natural killer of bacteria can offer alternative therapy for antibiotics. The study aimed to isolate and characterize a lytic bacteriophage against *P. aeruginosa* (clinically resistant strain/superbug) and to investigate its lytic potential to control bacterial planktonic cells and their biofilms on stainless steel surfaces. A lytic bacteriophage known as MAC-1 was isolated from wastewater against the selected *P. aeruginosa*-2750 strain. Its host range was moderate and it only infected six isolates. Phage MAC-1 was characterized through transmission electron microscopy and was classified to the Siphoviridae family. It has good heat and pH stability. Its latent time was 24 min with a burst size of about 410 virions per cell. The phage MAC-1 efficacy was determined against *P. aeruginosa* planktonic cells and biofilms on metallic surfaces. Isolated phage MAC-1 demonstrated promising activity against bacterial planktonic cells as well as in reducing bacterial biofilm biomass formed in 96-well and on stainless steel plates. However, a phage cocktail may be used to avoid resistance and ensure complete eradication of bacterial biofilms.

Keywords: Bacteriophage; biofilm; cocktail; planktonic cells; *Pseudomonas aeruginosa*

ABSTRAK

Pseudomonas aeruginosa ialah bakteria patogen yang boleh dianggap berisiko tinggi kepada kesihatan manusia kerana keupayaannya yang luar biasa untuk menentang antibiotik, sama ada secara intrinsik atau berikutan pemerolehan gen rintangan. *P. aeruginosa* telah dianggap sebagai ancaman utama kepada kesihatan manusia kerana tidak menunjukkan kesan daripada semua ubat sedia ada. Bakteriofaj sebagai pembunuh bakteria semula jadi boleh menawarkan terapi alternatif untuk antibiotik. Kajian ini bertujuan untuk mengasingkan dan mencirikan bakteria lisis terhadap *P. aeruginosa* (*superbug* rintang klinikal) serta mengkaji potensi lisisnya untuk mengawal sel planktonik bakteria dan biofilemnya pada permukaan keluli tahan karat. Bakteria lisis yang dikenali sebagai MAC-1 telah dipencilkan daripada air sisa terhadap strain *P. aeruginosa*-2750 terpilih. Julat perumahnya adalah sederhana dan hanya menjangkiti enam pencilan. Faj MAC-1 telah dicirikan melalui mikroskop elektron transmisi dan dikelaskan kepada famili Siphoviridae. Ia mempunyai kestabilan haba dan pH yang baik. Masa pendamnya ialah 24 minit dengan saiz letusan kira-kira 410 virion per sel. Keberkesanan faj MAC-1 ditentukan terhadap sel planktonik *P. aeruginosa* dan biofilem pada permukaan logam. Faj terpencil MAC-1 menunjukkan aktiviti yang memberangsangkan terhadap sel planktonik bakteria dan juga dalam mengurangkan biojisim biofilem bakteria yang terbentuk dalam plat 96-telaga dan pada plat keluli tahan karat. Walau bagaimanapun, koktel faj boleh digunakan untuk mengelakkan kerintangan dan memastikan pembasmian lengkap biofilem bakteria.

Kata kunci: Bakteriofaj; biofilem; koktel; *Pseudomonas aeruginosa;* sel planktonik

INTRODUCTION

Pseudomonas aeruginosa is considered an opportunistic Gram-negative bacterium (Santana et al. 2022). It is abundantly present in a wide range of natural environmental settings and may also be extracted from animals, humans, plants, and many other living sources (Groleau et al. 2022). Furthermore, *P. aeruginosa* is recognized as the third key driver for nosocomial urinary tract infections (UTIs). It is responsible for higher death rates because patients with other respiratory infections and immunocompromised systems are more susceptible (Chadha, Harjai & Chhibber 2022). *P. aeruginosa* is found to possess resistant characteristics against a diverse range of antimicrobial agents and hence falls in the category of superbugs (El-Telbany et al. 2022). *P. aeruginosa* has the ability to survive on minimal nutrients, even in harsh environmental conditions. This characteristic helps to foster bacterium proliferation in both hospitals, as well as natural environmental settings (Qin et al. 2022).

P. aeruginosa is also known to create specific structured entities under specific conditions, commonly known as 'biofilms' (Bobrov et al. 2022). Bacteria rely on a number of defense mechanisms to maintain their survival in severe and unfavorable environments for their normal life cycle (Karley, Shukla & Rao 2022). Biofilm development is the most fascinating process for developing increased resistance to destructive environmental factors (Figaj et al. 2023). A biofilm is a group of microorganisms grown on biotic and abiotic surfaces, in which various bacterial cells compactly adhere to each other embedded in extracellular polymeric substance (EPS) produced by the group of cells themselves (Zhang et al. 2023). The EPS is made up of polysaccharides, DNA and proteins (Das 2022). Biofilms are widespread within industrial and hospital surroundings, where they are available on non-living materials like surfaces of plastic, metals, and wood (Zhou et al. 2022).

Catheter-related infections are one of the most prevalent illnesses caused by biofilm-forming bacteria on medical devices, and they have been linked to increased patient morbidity and mortality rates (Di Domenico, Oliva & Guembe 2022). Phages possess the capability of reducing such infections; hence, catheter pre-treatment with a particular phage cocktail or phage lowered the chances of infections produced through biofilm (Chang et al. 2022). Phages have certain applications in modern biotechnology industries: they have been used as an alternative to antibiotics, as a carrier for transferring gene or protein vaccines, for detection of pathogenic bacteria, for targeted gene delivery and for screening peptides, antibodies and protein libraries (Qin et al. 2022). They also have a role in preventing biofilm formation on infectious wounds and surgical instruments. The key objective of this current study was to investigate the potential of phage therapy by isolating a fresh phage from wastewater and to demonstrate its application against multi-drug resistant (MDR) *P. aeruginosa* planktonic cells and biofilms formed on metallic surfaces.

MATERIALS AND METHODS

BACTERIAL AND WATER SAMPLING

There were 21 *P. aeruginosa* isolates collected from Army Medical College (AMC) Rawalpindi and Armed Forces Institute of Pathology (AFIP), Pakistan, during April-June 2016 in the form of culture plates. In both institutes, bacterial samples were isolated from wound swabs, blood, urine, and pus samples of patients. Bacterial strains were cultured and sub-cultured. For aid in future usage, the fresh glycerol stocks were stored at a temperature of -80 ºC. Concurrently, water samples were collected in sterilized containers from sewers and waste canals in different areas of Pakistan containing industrial and domestic wastewater bodies. The samples were stored at 4 ºC.

SELECTION OF BACTERIAL STRAINS

A total of 21 bacterial isolates were tested for biofilm formation ability using the 96-well plate by the method previously used by Jamal et al. (2015a) with some modifications. Each bacterium of interest was cultured in a culture tube (3-5 mL) overnight and grown up to the stationary phase. Then, the bacterial culture was 100 times diluted in LB media and incubated at 37 °C for 48 h in static incubators. On the following day, the planktonic bacterial cells and culture media were removed, and each well was washed out with distilled water. This step was repeated three times. In each well, 125 µL of crystal violet solution (1%) was added. The adhered cells were stained at room temperature for 10-20 min. The crystal violet solution was pipetted out, and the remaining solution was washed with distilled water. This step was repeated at least four times. After washing the crystal violet solution, 200 µL of dimethyl sulfoxide (DMSO) was added to each stained well. The optical density (OD) of each sample was measured using the ELISA plate reader at 500-600 nm.

IDENTIFICATION OF SELECTED BACTERIAL STRAIN

Gram staining was done to differentiate bacterial strains for Gram positive and negative. The bacterial strains were also allowed to grow on the MacConkey agar plate for further confirmation. By means of strain confirmation, the ribotyping was done by PCR amplification of 16S rRNA of *P. aeruginosa*-2750 using universal primers RS-3 and

RS-1 (Jamal et al. 2015b). For amplicon visualization, 1% of agarose gel was used. Purification of the PCR product was performed before sequencing of the DNA fragment was done. BLAST analysis of 16S rRNA was carried out for bacterial identification.

ANTIBIOTIC SENSITIVITY OF CLINICAL BACTERIAL ISOLATES

Following the recommendations of the 30th Clinical and Laboratory Standards Institute (CLSI) (2020), an antibiotic susceptibility test was carried out (Akram, Shahid & Khan 2007). The susceptibility of *Pseudomonas*-2750 was determined by the disc diffusion method. *T*o determine the antibiotic resistance of the selected bacterial strains, six different antibiotics were used (Table 1). On Mueller Hinton agar (MHA), a uniform swab of the strain was smeared on discs and petri dishes with different antibiotics, placed suitably distant from each other. Petri dishes were then allowed to incubate overnight at a temperature of 37 ºC.

ISOLATION AND PURIFICATION OF PHAGE MAC-1

Approximately 50 samples of wastewater were collected from diverse regions of Pakistan (April-July 2017) in order to isolate the phage against selected *P. aeruginosa*-2750 strain using previously reported methods by Jamal et al. (2015c) with some modifications.

DETERMINATION OF HOST RANGE

The collected strains of *P. aeruginosa* were evaluated for their sensitivity to bacteriophage in order to determine the host range of the isolated bacteriophage. The sensitivity of these strains was analysed through a spot test followed by a soft agar overlay, as illustrated by Sillankorva, Neubauer and Azeredo (2008).

THERMAL AND pH STABILITY OF PHAGE MAC-1

The phage heat stability was analysed by the method described by Noritomi et al. (2011), with some modifications. The phage filtrate was taken and incubated for a duration of 1 h at varying temperatures (37, 45, 55, 65, 70, and 80 °C). The pH stability was performed by the method previously reported by Capra, Quiberoni and Reinheimer (2006) with a few modifications. A diverse range of pHs (1, 3, 5, 7, 9, 11) were used in this study. After the incubation at a particular pH, each sample of bacteriophage was analyzed against host bacterial species by using the soft agar overlay technique.

CALCIUM AND MAGNESIUM IONS EFFECT ON PHAGE ADSORPTION RATE

The phages were treated with $MgCl_2$ or $CaCl_2$ in order to determine the cations influence on bacteriophage adsorption to host bacterium. By using the soft agar overlay technique, the free phages in each flask were determined (Capra, Quiberoni & Reinheimer 2006). The variations in the free phage titer within each flask showed the effect of $MgCl_2$ or $CaCl_2$ on phage adsorption. The following formula was applied for calculating the quantity of free phages, (where, N0 is PFU/mL at T = 0 min and N is PFU/mL at T= 10, 20 and 30 min, respectively).

ONE STEP GROWTH

The one-step growth method was used to determine the burst size and latent time period of the phage (Jamal et al. 2015d). In this method, 50 mL of bacterial culture with OD of 0.4-0.6 was centrifuged for cell harvesting. About 0.5 mL phage suspension (3×10^8 phages/cell) was added to the host bacterial liquid culture to determine the phage adsorption of bacterial cells. This mixture was centrifuged for 30 s at 14000 rpm to eliminate unabsorbed phages. The pellet was then suspended in 100 mL LB media again and incubated with constant shaking (120 rpm) at 37 ºC. After every 3 min, samples were collected from the flask, serially diluted and the number of phages (PFU) was calculated using the soft agar overlay/double agar method.

TEM ANALYSIS OF BACTERIOPHAGE

The morphology of the phage was analyzed by transmission electron microscopy (TEM), as previously described by Jamal et al. (2017). Ten-fold dilution of phage titer (approximately 10^{10} PFU/mL) was done within phage buffer having pH 6. A drop of 10^{10} PFU/mL of phage MAC-1 was put on the surface of a formvar-coated grid (200 mesh copper grids) that had been negatively stained with 2% uranyl acetate. The grid was analyzed using an electron microscope at 100 kV. Phage MAC-1 was morphologically classified according to the techniques and guidelines of the International Committee on Taxonomy of Viruses (ICTV) (Larson et al. 2003).

SUSCEPTIBILITY OF PLANKTONIC CULTURES TO THE PHAGE

Cerca, Oliveira and Azeredo (2007) established a technique for determining bacterial strain sensitivity to the phage. The cell suspension of the bacterial strain was adjusted to 2×10^8 cells/mL using normal saline before being introduced to flasks containing tryptone soya broth (TSB) medium. The bacteria were then incubated at 37 ºC with continuous shaking at 120 rpm until their density reached $>2\times10^8$ cells/

mL. In flasks containing *P. aeruginosa* culture, phage suspensions with varying multiplicity of infection (MOI) were introduced (0.0, 0.1, 0.5, 1.0 and 5.0). One flask was used as a control and did not contain any phages. All of these flasks were incubated for 5 h at 120 rpm and 37 ºC in a shaking incubator. Samples were collected from each flask after every hour and their OD was taken at 650 nm. The colony forming unit (CFU) was calculated according to the technique described by Jamal et al. (2017). The phage titer was analyzed throughout the bacterial growth by taking the sample after each hour interval following the serial dilution technique.

SUSCEPTIBILITY OF BACTERIAL BIOFILM TO PHAGE MAC-1

The bacterial biofilm was developed according to the method described by Cerca, Oliveira and Azeredo (2007). The bacterial overnight culture was diluted in TSB medium to 2×10^8 cells/mL at a 1:100 ratio before being placed in 96-well plates and covered with a lid. The bacterial culture was then incubated for 24 h in a shaking incubator (120 rpm) at 37 ºC. The planktonic bacterial culture was then collected and put into a collecting chamber containing a 10% bleach solution. Half of the wells were taken as controls, whereas in the other half, the phage was added with 1 MOI (4.5×10^9 PFU/mL). The wells were washed with distilled water after the removal of the crystal violet solution and placed inverted to dry. The OD_{600} of the samples was measured by the ELISA reader and the experiment was repeated three times (Adnan et al. 2020).

BIOFILM DEVELOPMENT ON STAINLESS STEEL PLATES AND TREATMENT WITH PHAGE

The evaluation of bacteriophage inhibitory potential on biofilm development was conducted according to Karaca, Akcelik and Akcelik (2015). For biofilm formation, static and dynamic conditions were used. The biofilm formation was studied using stainless-steel (SS) plates (2 cm × 2 cm) in a 6-well plate. For this purpose, SS plates were first washed with 100% acetone, followed by immersion in NaOH for about an hour. Then, the plates were rinsed with distilled water and 70% ethanol was sprayed on them. These plates were then placed in an incubator at 60 ºC for 1 hour, followed by autoclaving at 121 ºC for 15 min. Biofilm formation was allowed without the removal of media after 12 h. Biofilm formation on SS plates was studied at different time intervals.

SEM ANALYSIS OF BIOFILM

The biofilm formed on stainless steel (SS) plates was fixed using the same procedure with minor modifications (Sillankorva, Neubauer & Azeredo 2008). The dehydration process of the biofilm samples using the ethanol dilution consisted of concentration series ranging from 2% to 100%. The scanning electron microscope (SEM) analysis was then performed on the SS plates following the drying procedure. The SS plates with dehydrated biofilms were mounted on tabs of carbon adhesive on aluminium specimen mounts. The SS plates were rendered for conduction with carbon (Denton Desk II sputter coater). The SEM at the Institute of Space Technology (IST), Islamabad, Pakistan, was used for the examination and detection of biofilms in SS plates under both treatment and control conditions.

STATISTICAL ANALYSIS

The whole data has been expressed as means ± SD. For statistical analysis, MS Excel (version 2007) was used. The Student's *t*-test was carried out to find differences between treated and control (untreated) samples. The p value (≤0.05) was considered statistically significant.

RESULTS

DETECTION OF BACTERIAL BIOFILM

A total of 21 bacterial strains were tested for biofilm formation and measurement of OD at 650 nm showed that 7 strains formed moderate biofilms. Out of these strains, only one with efficient biofilm-forming ability was further studied to investigate the effect of phages on biofilm formation (Figure 1(A)).

BACTERIAL IDENTIFICATION

P. aeruginosa-2750 was identified as a Gram-negative rod-shaped bacteria. The identification of the bacterial strain was carried out through PCR, and a fragment of about 470 bp was obtained (Figure 1(B)). The amplicon was sequenced and subjected to BLAST to search for similar sequences. From BLAST analysis, it showed 100% nucleotide sequence identity to *P. aeruginosa.* The sequence was submitted to GenBank and assigned accession no. MH675483.

ANTIBIOTIC SENSITIVITY OF CLINICAL BACTERIAL STRAINS

The antibiotic susceptibility of *P. aeruginosa*-2750 was carried out through disc diffusion method. The selected bacterial strain showed resistance to amoxycillin (AM), tetracycline (TE), ampicillin (AMP), sulphamethoxazole/trimethoprim (SXT), erythromycin (E) and gentamycin (GM). The zone of inhibition for all antibiotics was 0 mm, except for gentamycin (9 mm) (Table 1).

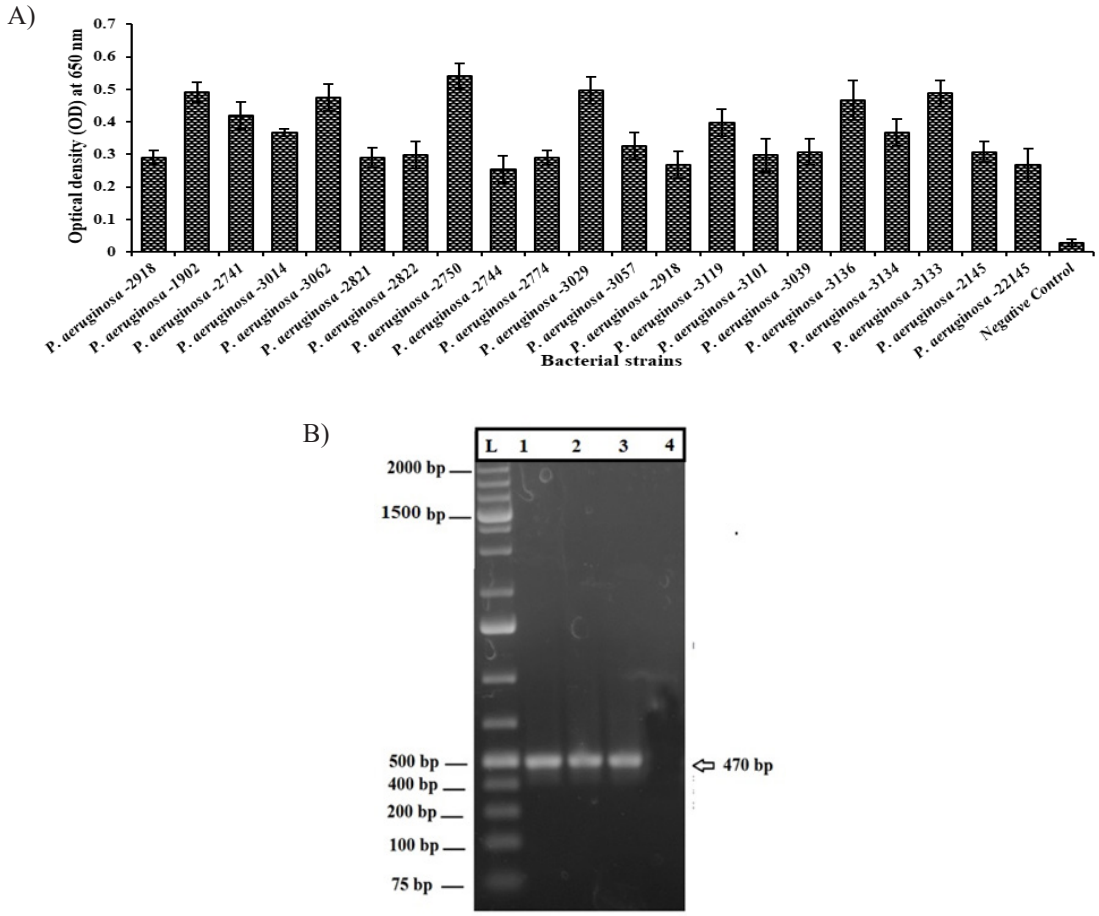


FIGURE 1. (A) Detection of biofilm formation. Only 7 strains formed moderate biofilm. Strain 8 (*P. aeruginosa*-2750) was selected for further characterization. (B) Bacterial strain identification by PCR ribotyping. Lanes 1-3: Amplification of the 16S rRNA gene (470 bp); Lane 4: Negative control; Lane L: Gene Ruler 1 kb Plus (Thermo Scientific) DNA ladder

TABLE 1. Antibiotic susceptibility of *P. aeruginosa*-2750 to different classes of antibiotics

Types of antibiotics	Generation	Concentration (µg)	Zone of inhibition (mm)	Resistant/ Susceptible
Amoxycillin (AM)	2nd	25	0	Resistant
Tetracyclin (TE)	1st	30	0	Resistant
Ampicillin (AMP)	-	25	0	Resistant
Sulphamethoxazole/ Trimethoprim (SXT)	-	25	0	Resistant
Erythromycin(E)	1st	15	0	Resistant
Gentamycin (GM)	2nd	10	9	Resistant

*The susceptibility and resistance of *P. aeruginosa*-2750 were determined according to the CLSI guidelines (2020)

ISOLATION OF PHAGES

The phage MAC-1 was isolated against *P. aeruginosa*-2750 from wastewater. The MAC-1 phage produced clear plaques ranging in size from 1 to 5 mm. Each phage showed plaques with well-defined boundaries (Figure 2).

HOST RANGE DETERMINATION

The host range of phage MAC-1 was examined using around 21 bacterial strains by spot test, followed by a soft agar overlay assay for further confirmation. Only six *P. aeruginosa* strains were susceptible to the lytic activity of MAC-1 phage (Table 2).

THERMAL AND pH STABILITY

The thermal stability test was carried out to check the temperature range of phage MAC-1. Results obtained showed that phage MAC-1 exhibited potential lytic activity after 1 h of incubation at 37 ºC but a gradual increase in temperature reduced the capability of the phage to survive, ultimately leaving no phage at 80 ºC. The phage was stable at temperatures ranging between 37 ºC and 60 ºC. The lowest activity was observed at 70 °C, whereas no activity was observed at 80 °C (Figure 3(A)). The optimum pH range for phage MAC-1 was determined by incubating phage MAC-1 at different pH ranges (1, 3, 5, 7, 9, and 11) for 15 h. The phage showed stability at pH 5, 7, 9 and 11, but not survived at pH 1 and 3 (Figure 3(B)).

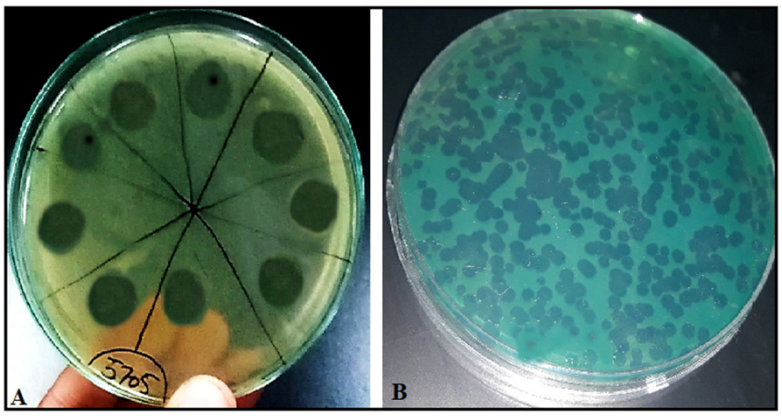


FIGURE 2. (A) The spot assay of bacteriophage MAC-1 against *P. aeroginosa*-2750. Clear spot was made by the bacteriophage on its host bacterial lawn, whereas (B) showed the soft agar overlay assay for bacteriophage MAC-1

TABLE 2. Host range of phage MAC-1 using spot test followed by plaque assay

Bacterial strain/ isolates	Phage activity (+/-)	Bacterial strain/ isolates	Phage activity (+/-)
P. aeruginosa 2750	+	*P. aeruginosa* 3057	-
P. aeruginosa 2918	+	*P. aeruginosa* 2918	+
P. aeruginosa 2902	+	*P. aeruginosa* 3119	-
P. aeruginosa 2741	-	*P. aeruginosa* 3101	-
P. aeruginosa 3014	-	*P. aeruginosa* 3039	-
P. aeruginosa 3062	-	*P. aeruginosa* 3136	-
P. aeruginosa 2821	-	*P. aeruginosa* 3134	-
P. aeruginosa 2822	+	*P. aeruginosa* 3133	-
P. aeruginosa 2744	+	*P. aeruginosa* 3130	-
P. aeruginosa 2774	-	*P. aeruginosa* 22145	-
P. aeruginosa 3029	-		

lysis (+), no lysis (-)

CALCIUM AND MAGNESIUM IONS EFFECT ON THE ADSORPTION RATE OF PHAGES

The effect of calcium (Ca^{2+}) or magnesium (Mg^{2+}) ions on phage adsorption was investigated by adding 10 mM $CaCl_2$ and $MgCl_2$ to the phage and host bacterial strain combinations independently. Using the plaque assay, the number of free phages (unbound) in the solution was determined at various time periods (0, 10, 20, and 30 min). There were substantial differences between the control and the phage MAC-1 treated with Ca^{2+} or Mg^{2+} ions (Figure 3(C)).

ONE STEP GROWTH

The one step growth technique was used to find the latent time and burst size of the phage MAC-1. The triphasic curve was obtained and it had log, stationary and latent phases. The burst size was about 410 phages per cell and the latent time period was 24 min (Figure 3(D)).

MORPHOLOGY OF PHAGE MAC-1

TEM analysis showed that phage MAC-1 had a round head of approximately 47 nm. It also had a long tail of about 114 nm with a width of 26 nm. On the basis of morphological features, MAC-1 was considered a phage belonging to the *Siphoviridae* family (Figure 4).

SUSCEPTIBILITY OF BACTERIAL PLANKTONIC CULTURES TO SPECIFIC PHAGE

The highly susceptible bacterial strain was treated with different MOIs of its respective host phage, MAC-1. The lytic activity of phage MAC-1 against the planktonic culture of *P. aeruginosa*-2750 in the exponential phase of its growth is illustrated in Figure 5(A). *P. aeruginosa* showed high susceptibility at all MOIs, but particularly at 5 MOI. Cell growth was observed by taking ODs at different time intervals, and the CFU count was also performed to calculate the actual bacterial count.

SUSCEPTIBILITY OF BIOFILM TO PHAGE MAC-1

To determine the action of phage on biofilm, 96-well cell culture plates were used to form biofilm for 24 and 48 h. Biofilm was then treated with MAC-1, and a reduction in biofilm was observed by taking the differences between ODs of treated and untreated biofilms. About 2.5 to 3.6-fold

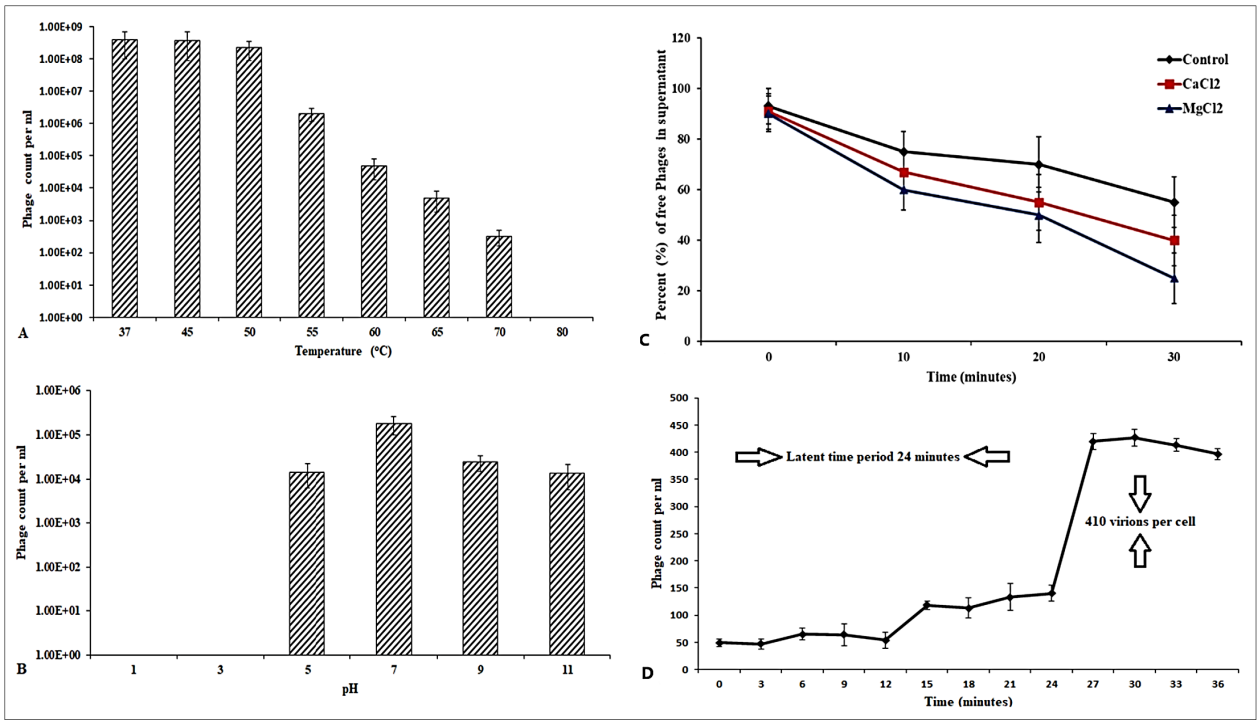


FIGURE 3. (A) Thermal stability test; (B) pH stability test; (C) Calcium and magnesium ion effect on the adsorption rate of phages; (D) One step growth analysis. All values are means ±SD (n = 3)

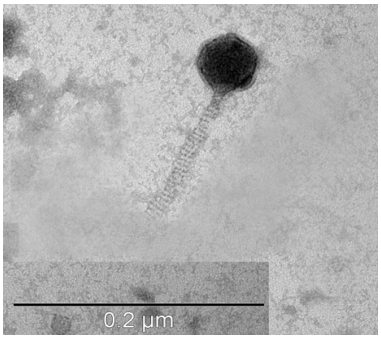
biomass reductions were observed in 24- and 48-h-old biofilms of *P. aeruginosa*-2750 after treatment with the phage titer of 4.5×10^9 PFU/mL. By paired sample *t*-test, a significant difference was observed in treated biofilm as compared to the control (untreated) (Figure 5(B)).

BACTERIAL BIOFILMS FORMED ON SS PLATES

P. aeruginosa-2750 biofilms were phage-treated for 4 h after being generated under static and dynamic conditions with no medium renewal for 24 and 48 h, resulting in biomass reductions of 4.3 log and 4.2 log, respectively (Figure 5(C), 5(D)). Phages were counted from the treated biofilms with the incubation time of 24 and 48 h. Significant reductions were observed in both 24- and 48-h periods when determined by paired samples *t*-test ($p<0.05$).

SEM ANALYSIS

Scanning electron microscopy (SEM) was performed for further confirmation of biofilm formation and observing the effect of phage MAC-1. Figure 6(A) and 6(B) shows the biofilm of P. aeruginosa-2750 without phage treatment whereas significant reduction biofilm of P. aeruginosa-2750 treated with MAC-1 can be seen in Figure 6(C) and 6(D).



Scale bar represents 200 nm

FIGURE 4. Electron micrograph of MAC-1 bacteriophage particle

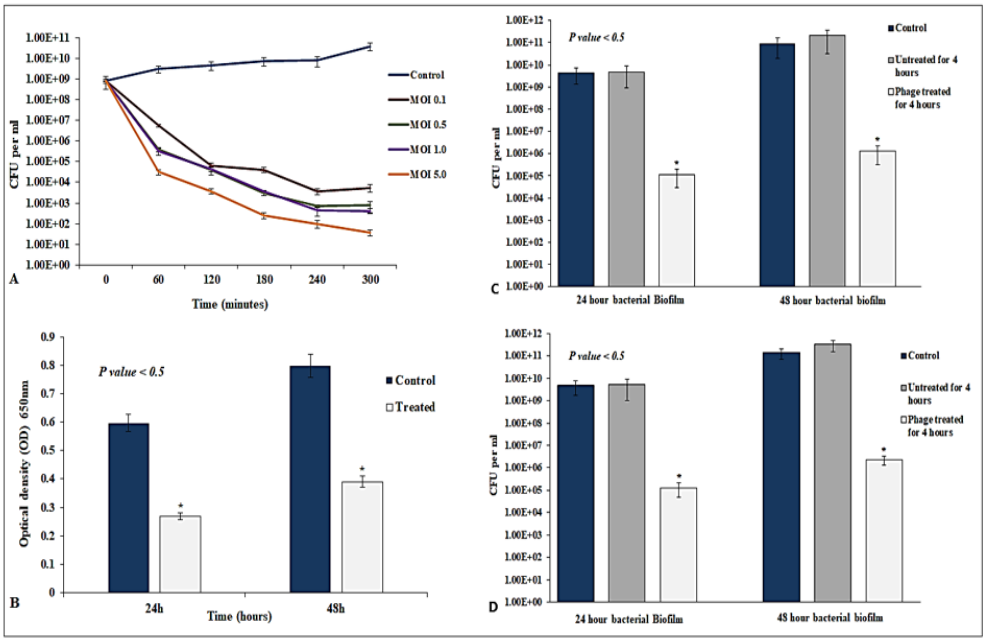


FIGURE 5. (A) Reduction curves of exponential growth phase planktonic P. aeruginosa-2750 infected with phage MAC-1 at different MOIs; (B) The susceptibility of biofilm to phage MAC-1. (C) Biofilm formed on SS plates under static conditions treated with phage for 4 h; (D) Biofilm formed on SS plates under dynamic conditions treated with phage for 4. All values are means ±SD (n = 3)

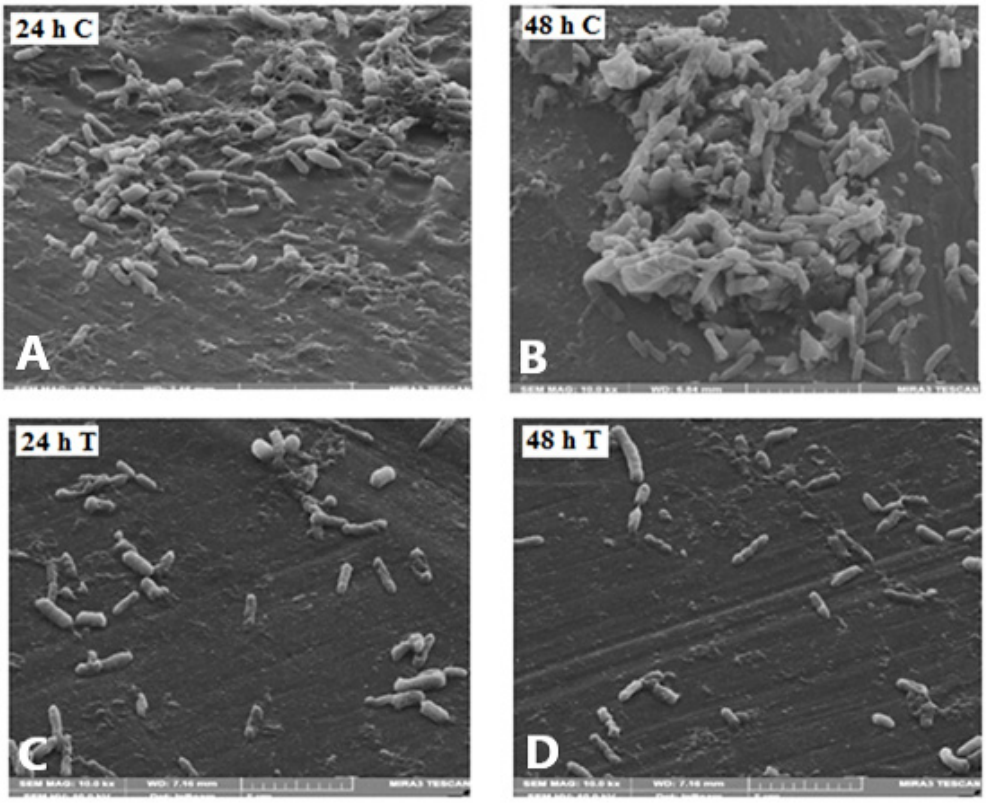


FIGURE 6. (A) indicates 24 h and (B) 48 h biofilms without phage treatment whereas (C) indicates 24 h and (D) 48 h biofilms after phage MAC-1 treatment for 4 h

DISCUSSIONS

P. aeruginosa is known to be an opportunistic pathogenic bacterium which is the main cause of infections in biofilm (Chadha, Harjai & Chhibber 2022). The development of antibiotic resistance in bacterial species strengthens the requirement to discover alternative strategies to manage these persistent biofilm infections (Qin et al. 2022). To control these resistant infections, researchers are trying to use bacteriophage remedy as a substitute to drugs. Phage therapy might be utilized for treating infections of both animals and plants (Lin et al. 2022). Bacteriophage remedy can also be applied for the healing of wounds that are unresponsive to antibiotic substances (Shukla et al. 2022). This study isolated a bacteriophage designated MAC-1 against MDR *P. aeruginosa*-2750 from wastewater. Phages can be frequently isolated against specific host bacterium from the environment (Chevallereau et al. 2022) since microorganisms are present in large quantities in the sewage water from homes and hospitals (Jamal et al. 2017).

The isolated phage was lytic and had a broad host range. The phage MAC-1 formed apparent plaques whose sizes were ranging from 1-5 mm in diameter. The phage host range may vary from narrow to broad (Hyman & Abedon 2010). MAC-1 showed a moderate host range and was capable of infecting *P. aeruginosa* 2750, *P. aeruginosa* 2918, *P. aeruginosa* 2902, *P. aeruginosa* 2822, *P. aeruginosa* 2744, and *P. aeruginosa* 2919 only. The narrow and moderate host range of MAC-1 may be due to *the development of exopolysaccharides, capsule formation, or bacterial resistance to phage adsorption, which has been reported for a number of bacteria (Labrie*, Samson & Moineau 2010). Similar findings from a phage typing experiment have been reported in Latino et al. (2016), where isolated mucoid phenotypes from lytic phage plaque edges showed resistance even when the phage was present.

The heat and pH stability of MAC-1 were also determined because the activity and survival of phages have a direct relationship with these factors (Ouellet et al. 2005). At pH 1 and 3, the phage was not active. According to Jamal et al. (2015d), phage therapy against bacterial infections will be more effective if the phages have high pH and heat stability. In this study, the isolated phages

show maximum stability at pH ranging from 5 to 9, suggesting that a wide variety of phages may survive in a wider range of ecological circumstances. The phage that we isolated exhibited stability even at the temperature of 70 °C and was comparable to Jamal et al. (2015e).

The isolated phage displayed enhanced adsorption to the bacterial host when it was treated with $MgCl_2$ or $CaCl_2$, (Figure 3(C)). The adsorption of phages to host bacteria is a function of the phage-bacteria relationship (Gill & Hyman 2010). The presence of magnesium or calcium chloride (5 mM) could increase phage adsorption to the bacterial host (Chhibber, Kaur & Kaur 2014), as Ca^{2+} ions play a role in supporting the adsorption of phages to bacteria (Russel et al. 1998). In this study, upon the addition of Ca^{2+} ions to the medium, a reduction in the quantity of free MAC-1 phages was observed, along with an associated stabilization in the adsorption process. These results were consistent with Jamal et al. (2017). Based on the one step growth curve assay, the phage MAC-1 burst size was about 410 virions per cell and its latent time was 24 min. This suggest that MAC-1 can be used in phage therapy.

P. aeruginosa-2750 was tested against seven different commercially available antibiotics and it was found resistant to all antibiotics (Table 1). At the exponential stage, the planktonic cells of bacterium displayed higher sensitivity to bacteriophage MAC-1 as compared to the control, and this was described previously on the planktonic cultures of *P. fluorescens* (Sillankorva et al. 2004). We did not notice a total eradication of biofilm, however, about 2.5 to 3.6-fold biomass reductions in 24- and 48-h biofilm of *P. aeruginosa* 2750 after treatment with phage titer of 4.5×10^9 PFU/mL were observed. This suggests that one bacteriophage only may not be capable of completely eradicating or treating the biofilm of bacteria. Thus, a bacteriophage cocktail (combination of two of more phages) may be the best way to treat or control the biofilm formed by bacteria. Martins et al. (2022) have reported excellent inhibition of the growth of the majority of extreme drug resistant (XDR) *K. pneumoniae* ST16 strains isolated from different countries by using phage cocktail. Similarly, phage cocktail has also shown tremendous activity in both *in vitro* growth inhibition assays as well as *in vivo* assays using *Galleria mellonella* model (Jeon, Park & Yong 2019).

A clinical strain of *P. aeruginosa*-2750 biofilm was developed in the laboratory on a Grade B SS plate under dynamic and static conditions. MAC-1 phage that was previously isolated and characterized was applied on the biofilm as a treatment for its reduction. However, a slight difference has been observed in biofilm reduction under both conditions. It suggests that the MAC-1 phage was found to possess a specific enzyme that is required for biofilm reduction (Fu et al. 2010).

CONCLUSIONS

In this study, the phage MAC-1 showed remarkable efficacy in controlling and reducing biofilms. The results indicated that the phage MAC-1 disrupts and reduces the formation of biofilms, highlighting its potential as an effective biofilm control agent. MAC-1 is quite stable under varying temperature and alkaline pH concentrations. Likewise, it showed a latent time period of 24 min and burst size of about 410 virions per cell, which are potentially used for phage therapy. It showed strong activity against host bacteria planktonic cells as well as against the biofilm of the host bacterium. By reducing the possibility that bacterial strains would become resistant to a particular phage, cocktail strategies could improve the overall effectiveness of biofilm control efforts.

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